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INTERACTIONS OF FLUORESCENT ANALOGS OF ADENINE NUCLEOTIDES WITH PYRUVATE KINASE

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1. Introduction

In view of the central role played by adenine nucleotides in metabolism, fluorescent analogs of ADP and ATP represent potentially valuable probes of enzymic mechanism and structure [1, 2]. Since the fluorescence properties of 1, N^6-ethenoadenosine di- and triphosphate (ϵADP and ϵATP)* are particularly favorable for a variety of physical studies, we elected to study the kinetic and fluorescence behavior of these analogs in detail with the enzyme pyruvate kinase. We have now shown that ϵADP and ϵATP substitute very well for ADP and ATP in terms of reaction rates. Moreover, the binding of ϵADP and ϵATP to the enzyme can be observed by fluorescence polarization techniques.

2. Materials and methods

All chemicals used were of reagent grade or the highest purity available commercially. Lactate dehydrogenase rabbit muscle (EC 1.1.1.27) was purchased from Boehringer-Mannheim. Rabbit muscle pyruvate kinase (EC 2.7.1.40) used in early studies was from Boehringer-Mannheim, while that used in final studies was purified according to Tietz and Ochoa [3] from frozen rabbit muscle purchased from Pel-Freez Biologicals. The purified enzyme had a minimum specific activity of 335 units/mg protein, where 1 unit is defined as 1 μmole product formed per min as measured in the following coupled assay: 100 mM tetramethylammonium-*N*-Tris-(hydroxymethyl) methyl-2-aminoethane sulfonic acid buffer, TMA-TES (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 5 mM ADP, 0.2 mM NADH, and excess lactate dehydrogenase in a final vol of 1 ml. The reaction was followed on a Gilford 240 recording spectrophotometer, thermostated at 26°. All kinetic measurements of the forward reaction were made under these conditions, except where 10 mM $MnCl_2$ was substituted for $MgCl_2$, the buffer used was 50 mM Tris (pH 7.5).

Pyruvate enolization was measured by following the detritiation of 3-T-pyruvate (prepared by the procedure of Meloche [4]) under the following conditions: 100 mM TMA TES (pH 7.5, 100 mM KCl, 100 mM Na 3-T-pyruvate (20,000 cpm/μmole), 37 μg pyruvate kinase, and ATP and divalent metal ion as indicated, in a total vol of 0.5 ml. Incubations were for 30 min at 26°, and water was distilled and counted as described by Robinson and Rose [5].

Fluorescence measurements were made on the instrument described by Weber and Young [6]. The fluorescence polarization measurements were carried out on the instrument described by Weber and Bablouzian [7]. The light source was a 450-Watt xenon arc cooled by air draft. The 310 nm exciting light was selected by a Bausch and Lomb grating monochromater. The cuvette holder fits square cuvettes of 1 cm internal dimensions. Brass adaptors were used to accomodate square cuvettes of 2 mm and 1 mm internal dimensions. The emissions were filtered through 2 mm of 0.1 M $NaNO_2$ and Corning Glass CS 3-75 filters. A Dana Digital Voltmeter was used to obtain the ratio of

* In the abbreviations ϵADP and ϵATP, ϵ stands for the etheno bridge and is also suggestive of the molar absorbance term and of fluorescence emission.

$I_{\parallel}$ to $I_{\perp}$, where $I_{\parallel}$ is the intensity of the emission light polarized parallel to the exciting beam and $I_{\perp}$ is the intensity of the emission light polarized perpendicular to the exciting beam. $I_{\parallel}/I_{\perp}$ measurements were averaged by using an average-and-standard deviation program on a Hewlett-Packard 9100A calculator. The polarization was calculated as

$$P = \frac{I_{\parallel}/I_{\perp} - 1}{I_{\parallel}/I_{\perp} + 1} .$$

All fluorescence measurements were made at 4°. When measured in the presence of $MgCl_2$ (10 mM), the solution contained 50 mM Tris (pH 8.5) and 100 mM KCl. With $MnCl_2$ (10 mM) as the divalent ion, the solution contained 50 mM Tris (pH 7) and 100 mM KCl. The enzyme was dialyzed extensively against the final solution before fluoresence measurements were made; the enzyme lost up to 1/2 of its activity upon dialysis. For protein determination the extinction coefficient of 0.54 ml mg^{-1} cm^{-1} at 280 nm was used [8]. The molecular weight of the enzyme is 237,000 [9] and it appears to be composed of 4 identical subunits [10].

Equilibrium dialysis experiments were conducted in lucite microdialysis cells [11] with a capacity of 0.5 ml/sector. Visking 20/32 dialysis tubing (Union Carbide) was used. The concentrations of the ligands in samples were determined from the absorbance at 310 nm. The filled dialysis cells were placed on a rotor at 4° with a rotational speed of about 8 rpm.

Table 1
Kinetic constants of ϵADP and ϵATP with pyruvate kinase.

Analog	Reaction	Divalent ion	K_m*(mM)	V_{max}**
ϵADP	Forward velocity	Mg^{2+}	0.30 (0.30)	0.80
ϵADP	Forward velocity	Mn^{2+}	0.25 (0.25)	0.41
ϵATP	Pyruvate enolization	Mg^{2+}	0.60 (0.60)	0.67
ϵATP	Pyruvate enolization	Mn^{2+}	0.50 (0.20)	0.68
ϵATP	Inhibitor with respect to phosphoenolpyruvate	Mg^{2+}	$\frac{K_i}{2.2}$ (1.5)	

* The K_m for normal substrate (ADP or ATP) is shown in parenthesis.
** Relative to normal substrates.

3. Results and discussion

3.1. Kinetic behavior of ϵADP and ϵATP

The apparent Michaelis constant (K_m) and relative maximum velocities (V_{max}) of ϵADP and ϵATP in the forward reaction and in the enolization of pyruvate, respectively, were examined. The velocities responded to concentration of analog in the normal hyperbolic fashion, as evidenced by linear Lineweaver–Burke plots. The kinetic constants were determined for the normal substrate and its analog under identical conditions. Table 1 lists the kinetic constants with both Mg^{2+} and Mn^{2+} as the required divalent metal ion. The values for K_m and V_{max} of the normal substrate and fluorescent analog do not differ greatly. The analog mimics, therefore, the behavior of the normal substrate in its interaction with enzyme during the catalytic reaction. Accordingly, its properties can be related to those of the normal substrate. The inhibition by ϵATP with respect to PEP was also examined and found to be of the competitive type. The K_i values for both ATP and ϵATP are observed to be comparable (table 1). It will be remembered, as emphasized by Reynard [12], that since ATP is a competitive inhibitor with respect to both PEP and ADP, the observed K_i with respect to one substrate does not correspond to the dissociation constant of ATP from the enzyme. The best values for the dissociation constant of substrates and products from pyruvate kinase are those derived from proton relaxation rates and kinetic protection by Mildvan and Cohn [13].

3.2. Fluorescence behavior of ϵADP and ϵATP

When a fluorescent molecule binds to a macromolecule which rotates more slowly than does the free "dye", then the fluorescence polarization of the dye increases†. In a mixture of protein and ligand, where part of the ligand is bound to the protein and the remainder is free, the observed polarization will be the average of the polarization of the free ligand and the ligand bound to the protein, weighted according to their contributions to the intensity. From the relative

† We shall refer to small fluorescent molecules (ϵADP or ϵATP) as ligands, using the general terminology for molecular complex formation.

Table 2
Fluorescence polarizations of ligand (ϵADP or ϵATP)-pyruvate kinase.

Ligand	Concentration of ligand (M)	Concentration of pyruvate kinase (M)	Divalent ion	$I_{\parallel}/I_{\perp}$	P
ϵADP	5.0×10^{-5}	0	Mg^{2+}	0.9779	−0.0112
ϵADP	5.0×10^{-5}	5.3×10^{-5}	Mg^{2+}	1.0084	0.0042
ϵADP	1.0×10^{-4}	5.3×10^{-5}	Mg^{2+}	1.0108	0.0053
ϵADP	1.5×10^{-4}	5.3×10^{-5}	Mg^{2+}	1.0000	0.0000
ϵATP	5.0×10^{-5}	0	Mg^{2+}	0.9779	−0.0112
ϵATP	5.0×10^{-5}	5.3×10^{-5}	Mg^{2+}	1.0076	0.0042
ϵADP	2.0×10^{-4}	0	Mn^{2+}	0.9774	−0.0114
ϵADP	2.0×10^{-4}	1.1×10^{-4}	Mn^{2+}	1.0651	0.0315
ϵADP	6.4×10^{-4}	1.0×10^{-4}	Mn^{2+}	1.1014	0.0482
ϵADP	1.0×10^{-3}	9.1×10^{-5}	Mn^{2+}	1.0908	0.0434
ϵADP	2.0×10^{-3}	7.0×10^{-5}	Mn^{2+}	1.0772	0.0371
ϵATP	2.0×10^{-4}	0	Mn^{2+}	0.9773	−0.0115
ϵATP	2.0×10^{-4}	1.1×10^{-4}	Mn^{2+}	1.1142	0.0540
ϵATP	6.4×10^{-4}	1.0×10^{-4}	Mn^{2+}	1.1440	0.0672
ϵATP	1.0×10^{-3}	9.1×10^{-5}	Mn^{2+}	1.1282	0.0602
ϵATP	2.0×10^{-3}	7.0×10^{-5}	Mn^{2+}	1.0644	0.0311
ϵADP	1.0×10^{-3}	9.1×10^{-5}	Mn^{2+}	1.1426	0.0666[a]
ϵATP	6.4×10^{-4}	1.0×10^{-4}	Mn^{2+}	1.1914	0.0873[a]
ϵATP	1.0×10^{-3}	9.1×10^{-5}	Mn^{2+}	1.1935	0.0882[a]
ϵATP	2.0×10^{-3}	7.0×10^{-5}	Mn^{2+}	1.1358	0.0636[a]

[a] In a 50% sucrose solution.

fluorescence yields of the ligand, free and bound, and the fluorescence polarization of the ligand–protein system, the amount of ligand bound can be calculated [14]. Using these principles we can describe the binding behavior of ϵADP and ϵATP to pyruvate kinase.

When pyruvate kinase was added to ϵADP or ϵATP solutions, the polarizations increased but were still less than 0.01 in the ϵADP–pyruvate kinase–Mg^{2+} system. When the divalent activator was changed from magnesium to manganese, the polarizations of the ligand–protein systems increased to a few percent which were significant and reproducible, but those of the free ligands were not affected. This suggests that Mn^{2+} ion increases the binding of ϵADP and ϵATP to pyruvate kinase. The observed low polarizations for the ligand–protein system (table 2) could be due to the low binding affinity of the ligands to the protein or to the ability of the bound ligand to undergo rotational depolarization. Equilibrium dialysis experiments were carried out, and the concentrations of the free and the

Table 3
Results from equilibrium dialysis.

Ligand	Free ligand concentration at protein site (M)	Bound ligand concentration at protein site (M)	Concentration of pyruvate kinase (M)	Divalent ion	$I_{\parallel}/I_{\perp}$	P
ϵADP	7.32×10^{-4}	2.07×10^{-4}	1.1×10^{-4}	Mn^{2+}	1.0842	0.0404
ϵADP	6.95×10^{-4}	2.60×10^{-4}	1.1×10^{-4}	Mn^{2+}	1.3530	0.0634

Total ligand concentration was 1.67×10^{-3} for both ϵADP and ϵATP.

bound ligand as well as the polarizations of the systems were determined. If we use the fluorescence polarization (~0.34) of 1, N^6-ethenoadenosine in propylene glycol at −50° when excited at 310 nm [2] as the limiting polarization of the ligand–protein conjugates (for both ϵADP and ϵATP), a rough calculation shows that in the equilibrium dialysis experiments, the observed polarization of ϵADP-pyruvate kinase in the presence of Mn^{2+} should be about 0.07 and that of ϵATP-pyruvate kinase about 0.09, both higher than the observed values (table 3). This indicates that rotational depolarization is the major cause of the observed low polarizations for the ligand–protein systems. When sucrose was added to the protein–ligand systems to increase the viscosities of the solutions the polarizations increased about 30% and were closer to the estimated limiting values (table 2). These data eliminate the assumption that the depolarization is mainly due to protein rotations because the rotational rate of such a large molecule as pyruvate kinase (M.W. = 237,000) cannot be affected that drastically in a 50% sucrose solution. According to the results observed, ϵADP and ϵATP, as surrogates for ADP and ATP, bind to the protein in such a way that the fluorophor portion can rotate somewhat freely in the solutions. Accordingly, the base part of the ligand is not strongly associated with the protein through multiple points of attachment. This observation and the enzyme kinetic results corroborate the known broad specificity [15] and the proposed binding models [13] of pyruvate kinase for nucleotide substrates as obtained from other data.

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